

## Fluorescent Method for the Detection of Excreted Ribonuclease Around Bacterial Colonies

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### ABSTRACT

LANYI, JANOS K. (Stanford University School of Medicine, Palo Alto, Calif.), AND JOSHUA LEDERBERG. Fluorescent method for the detection of excreted ribonuclease around bacterial colonies. *J. Bacteriol.* **92**:1469–1472. 1966.—A test for the release of extracellular ribonuclease by *Bacillus subtilis* colonies was developed. The method consists of incorporating acridine orange and ribonucleic acid into nutrient agar plates and viewing the grown bacterial colonies under ultraviolet light. Regions of ribonuclease secretion appear as dark halos around the colonies on a green fluorescent background. The theoretical basis and the utility of this test are discussed.

Nucleases are released into the growth medium (3, 4, 9, 11, 16) by many species of bacteria. The genetic study of these extracellular enzymes has been hindered by the limitations of existing methods either for detecting deviant colonies on agar plates or for observing the time of enzyme excretion during growth. In one method (5), the bacteria were grown on agar plates containing large amounts of nucleic acid; the areas of nuclease action around the colonies were detected, upon flooding the plates with hydrochloric acid, as clear halos amidst the precipitated nucleic acid. The procedure kills the bacteria. This hindrance applies also to another method (1) where a phosphate reagent is used in combination with trichloroacetic acid. Another technique for the detection of deoxyribonuclease (15) is based on a color difference between toluidine blue and its complex with deoxyribonucleic acid (DNA).

It has been our intention to make use of the high sensitivity of fluorescence assay in extracellular enzyme detection. Our efforts were directed toward the extracellular ribonuclease of *Bacillus subtilis* Marburg strain (6, 9). According to previous reports, when a fluorescent dye, such as acridine orange, is combined with a large excess of polymeric ribonucleic acid (RNA) a complex is formed (2, 13) in which the dye molecules are intercalated between successive layers of the base pairs (8). This interaction enhances the green fluorescence of the dye. We expected that on depolymerization by nuclease the complex would be destroyed and the fluo-

rescence would be decreased. The zones of nuclease action around the bacterial colonies would be detected in this way as dark halos on a green fluorescent background.

### EXPERIMENTAL

Acridine orange is known to have an emission peak at 540 m $\mu$  which is enhanced up to three-fold by adding a large excess of nucleic acid polymers (2, 13). This effect was explored by taking the fluorescence spectrum of a solution of acridine orange and RNA with a Zeiss RMQ spectrofluorometer. The exciting light used was broad-band ultraviolet, and the spectrum of the emitted light was recorded manually at the highest sensitivity setting. The concentration of acridine orange was  $2.5 \times 10^{-4}$  M; that of the RNA (Calbiochem) was  $2.5 \times 10^{-3}$  M (in nucleotide) at pH 7.0. The results obtained are shown in arbitrary fluorescence units versus wavelength in Fig. 1, where it can be seen that the addition of a 10-fold excess of RNA does enhance fluorescence without changing the shape of the emission spectrum. Even though the increase in fluorescence became greater upon manipulating the relative concentrations of dye and nucleic acid, this effect would not be great enough by itself for a visual ribonuclease assay. However, substituted polysaccharides such as agar interact with free acridine orange (14) and the fluorescence of the dye is quenched. The quenching effect is shown by adding 0.35% agar (kept in solution before mixing by holding the temperature between 40 and 50 C) to a solution of acridine orange ( $2 \times 10^{-4}$  M) and recording the emission

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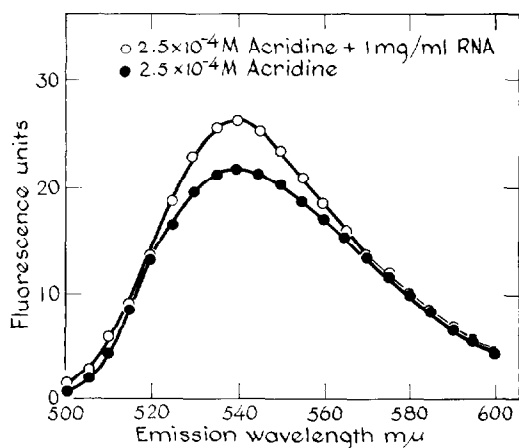


FIG. 1. Fluorescence of acridine orange in the presence and absence of RNA.

spectrum. The results (Fig. 2) indicate that in the presence of agar the fluorescence is almost completely eliminated.

The mechanism of this quenching is not understood. To explore the possibility that an electrolyte-type binding between the dye and agar plays a part, a solution of acridine orange ( $2 \times 10^{-4}$  M) was titrated by agar to the point of quenching. In Fig. 3, the extent of quenching is plotted as a function of the concentration of agar. It is seen that quenching is a linear function of the dose of agar up to a point of saturation. For calculating the stoichiometry of the interaction, one needs to know the number of ionizable groups in agar and on the acridine orange molecule. It has been reported that agar contains one sulfate ester group for approximately 53 galactose units (10). Acridine orange, on the other hand, has three amino groups per molecule. The equivalence point in this case, based on the assumption that all three amino groups are charged and can bind the sulfate groups, is at about 0.075% agar. The curve in Fig. 3 indicates that this is indeed the agar concentration where quenching approaches a maximum. A quenching curve for acridine yellow is also given, with similar results.

It should be noted that, even though the dependence of quenching on agar concentration is linear, it does not represent a 1:1 relationship; the slope varies for different dyes. No explanation is advanced for this discrepancy at this time. Nevertheless, it is tentatively concluded that the quenching of fluorescence by agar is a result of electromeric interaction between the dye and the agar.

Complexes of acridine orange with agar are

weaker than with nucleic acid, and the latter therefore reverses the quenching. To show this, a 10-fold excess of RNA was added to the acridine orange-agar complex, and the fluorescence spectrum was recorded. The results, shown in Fig. 2, bear out the expectation; it can be seen that fluorescence is fully restored by addition of RNA. This increase in the fluorescence of the acridine orange, due to the presence of RNA, is quite dramatic, and proved suitable for visual detection.

Preliminary to the agar-plate experiments, the RNA-acridine orange-agar complex was tested as an assay system for ribonuclease in liquid state. The assay mixture consisted of 1.0 ml of acridine orange-RNA ( $10^{-3}$  M dye,  $10^{-2}$  M RNA neutralized) and 3.0 ml of agar solution (0.05%). This mixture exhibited a fluorescence of 35.8 arbitrary units as compared with a control

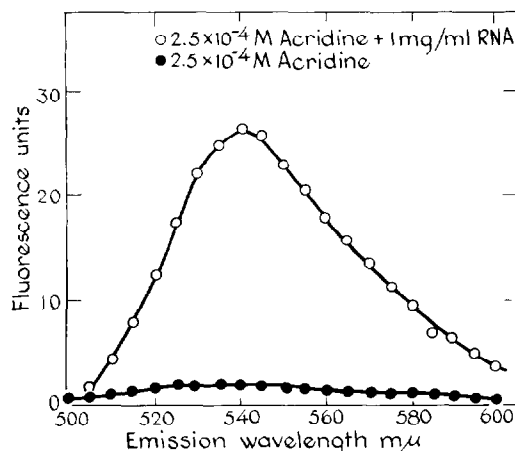


FIG. 2. Fluorescence of acridine orange in 0.35% agar solution in the presence and absence of RNA.

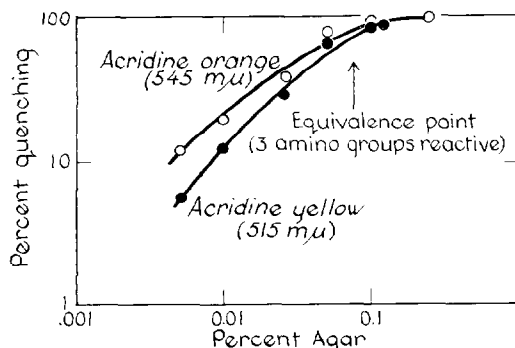


FIG. 3. Quenching of acridine orange and acridine yellow fluorescence by agar (as determined at the indicated wavelengths).

where distilled water was substituted in place of the agar solution, and where the fluorescence was 37.8 units. In two subsequent experiments, 10 and 20  $\mu$ liters of pancreatic ribonuclease solution [2.0 mg/ml in tris(hydroxymethyl)-aminomethane-HCl buffer, pH, 5 Worthington Biochemical Co., Freehold, N.J.] was added, and the change in fluorescence was observed. The solution was kept at room temperature. The two kinetic curves obtained are shown in Fig. 4. It is clear from this graph that the amounts of enzyme and the slopes observed for the decrease in fluorescence are not strictly proportional. However, as our intention was merely to develop a semiquantitative ribonuclease assay, the approximate correlation obtained was considered satisfactory. It has been reported that deoxyribonuclease is competitively inhibited by acridine orange (7). However, as the concentration of the dye needs to be severalfold higher for 50% reduction in activity than that used here, this effect was not considered important.

Agar plates for extracellular ribonuclease detection were made up in the conventional way with the following exception. Before autoclaving, 1 ml of stock solution (1 mg/ml of acridine orange and 15 mg/ml of RNA, neutralized with 1 N NaOH) was added to 100 ml of medium.

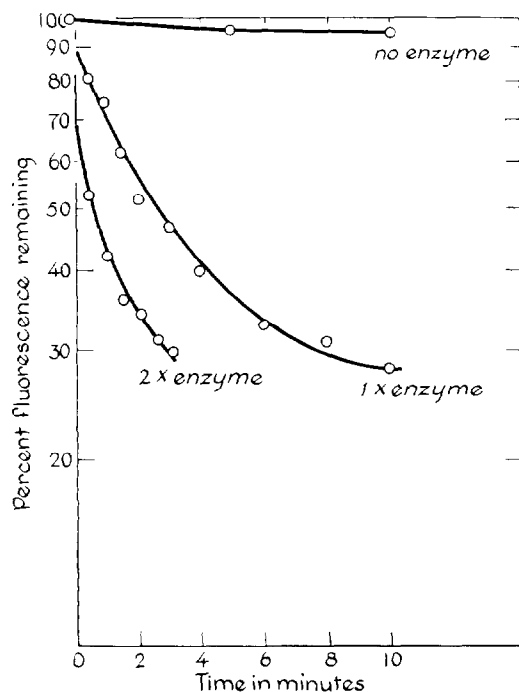


FIG. 4. Decrease in the fluorescence of the acridine orange-RNA-agar complex on addition of pancreatic ribonuclease.

After autoclaving for 20 to 25 min at 120 C, the medium was left to cool at 50 C for at least 2 hr. This latter period was necessary for obtaining maximal fluorescence. Upon solidifying, the plates were viewed with an ultraviolet lamp (Mineralite) in the dark; intense green fluorescence was observed.

*B. subtilis* colonies grew normally on these plates; cells picked from the surface of the agar proved viable. The action of various *B. subtilis* strains upon the acridine orange-RNA plates was then studied. The full details of obtaining these strains are being published elsewhere (Lanyi and Lederberg, *in preparation*); suffice it to say here that they were produced by ultraviolet irradiation of the *B. subtilis* Marburg strain and subsequent plating on fluorescent plates. The objective of this study was to establish a correlation between the size of dark (non-fluorescent) halos around the colonies and another, more quantitative, assay of ribonuclease activity performed on the culture filtrates of the same strains. The halo-forming property of four strains of *B. subtilis* is shown in Fig. 5. The agar medium consisted of a minimal medium described by Spizizen (12): 1% agar, 0.05% acid casein hydrolysate (neutralized; Nutritional Biochemicals Corp., Cleveland, Ohio), and 20  $\mu$ g/ml of L-tryptophan. The photographs in Fig. 5, taken 18 to 20 hr after streaking, show that, on this agar medium, the size of the halos

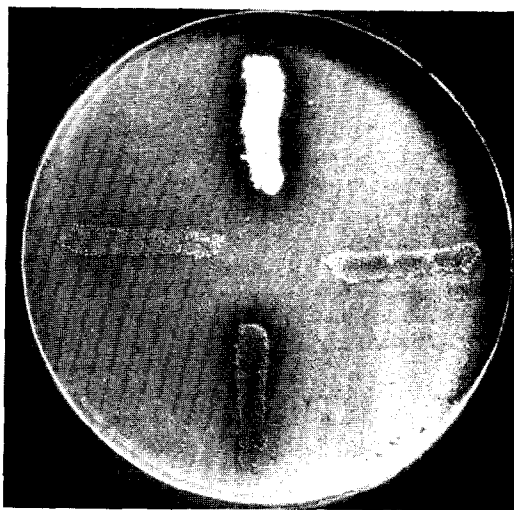


FIG. 5. Halo production by several strains of *Bacillus subtilis*. The agar plate was made and the bacterial colonies were grown according to the procedure described in the text. The plate was photographed in ultraviolet light with a yellow-green filter. Strains (clockwise, starting at top): SB 168, SB 764, SB 765, SB 29.

TABLE 1. *Halo formation and ribonuclease secretion by some strains of Bacillus subtilis*

Strain	Halo size	Net counts per 5 min	RNA hydrolyzed <i>μ</i> moles
SB 168.....	++++	1,817	20.6
SB 29.....	++	803	9.1
SB 764.....	- +	319	3.6
SB 765.....	+++	1,428	16.2

varies with the strains; such differences were found to be reproducible.

The strains used above were also grown in a liquid medium similar to that described above, containing the following: inorganic salts (12), 0.8% yeast nitrogen base (without amino acids; Difco), 0.1% acid casein hydrolysate (neutralized; Nutritional Biochemicals Corp.), 4% soluble starch, and 20  $\mu$ g/ml of L-tryptophan. The cultures were grown at 30 C with shaking. At 124 hr., the samples were withdrawn and centrifuged; the supernatant fluid was assayed for ribonuclease with tritiated RNA as substrate. The assay mixture contained 20  $\mu$ liters of  $H^3$ -uridine RNA ( $6.4 \times 10^4$  counts per min) in 0.2 ml of 0.05 M glycine-NaOH buffer (pH 9.55), to which 0.1 ml of culture supernatant fluid was added. After incubation at 37 C for 30 min, the samples were chilled, and 0.3 ml of high molecular weight bacterial RNA (1.25 mg/ml) was added. The unhydrolyzed portion of the nucleic acid was precipitated at 0 C by adding 1.0 ml of 7% trichloroacetic acid; the soluble fraction was collected after centrifugation, and 50  $\mu$  liters was counted in a Packard Tricarb scintillation counter. Zero-time digests gave 400 to 500 counts per 5 min; these values were subtracted from the experimental data. The results obtained in these assays are given in Table 1. Also given in Table 1 is the visual estimate of the size of the halos for the various strains. There was a good correlation between the results of the fluorescence assay and the results obtained through the more conventional assay described above. It was concluded, therefore, that the fluorescent plate technique can be used successfully in detecting differences in the levels of extracellular ribonuclease production. The method was found to have the advantage that large numbers of colonies (up to 100 to 150 per plate) can be examined at a glance, and mutants can be easily recognized and isolated.

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